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The role of lipocortin-1 in dexamethasone-induced suppression of PGE_2 and TNFα release from human peripheral blood mononuclear cells

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1 Lipocortin-1 and its N-terminal derivatives exert potent inhibitory actions in various models of acute inflammation. The present study examined the ability of lipocortin (LC)-1 to suppress the release of the acute pro-inflammatory mediators, tumour necrosis factor (TNFα) and prostaglandin E_2 (PGE_2) from human peripheral blood mononuclear cells (PBMC) stimulated with lipopolysaccharide (LPS) or recombinant human interleukin-1β (rhIL-1β).

2 LPS (10 μg ml^{-1})-stimulated release of TNFα and PGE_2 from PBMC was significantly inhibited by (4 h) co-incubation of the cells with 10^{-6} M dexamethasone (Dex), but not with 10^{-9} M to 10^{-7} M of a N-terminal fragment (amino acids 1–188) of recombinant human LC-1 (LC-1 fragment). However, Dex suppression of LPS-stimulated TNFα and PGE_2 secretion from PBMC was reversed when polyclonal antibody to LC-1 fragment (1 : 10,000 dilution) was included in the medium. rhIL-1β (5×10^{-8} M)-stimulated release of TNFα and PGE_2 from PBMC (after 18 h) was abolished by co-incubation of the cells with 10^{-7} M LC-1 fragment.

3 After incubation with Dex (4 h), cellular proteins from PBMC were immunoblotted using anti-LC-1 fragment antibody (which showed no cross-reactivity with human annexins 2 to 6). Dex caused no increase in immunoreactive (ir)LC-1 content of PBMC, although there was a three fold increase in the amount of a lower mass species with LC-1-like immunoreactivity. This was accompanied by the appearance of irLC-1 in the extracellular medium.

4 The results of the present study implicate endogenous LC-1 in glucocorticoid suppression of TNFα and PGE_2 release from human PBMC and suggest an extracellular site of action for LC-1. LC-1 may also inhibit rhIL-1β-stimulated TNFα and PGE_2 secretion from PBMC.

Keywords: Lipocortin-1; annexin-1; tumour necrosis factor α; prostaglandin E_2; monocyte

Introduction

Lipocortin-1 (LC-1), was originally identified in leukocytes as a glucocorticoid-inducible protein which potentially inhibited phospholipase A_2 (PLA_2) activity, and thus prevented pro-inflammatory eicosanoid generation (Flower, 1988). LC-1 is now known to belong to a family of structurally-related proteins, the lipocortins (or annexins), which bind phospholipid membranes in a calcium-dependent manner (Raynal & Pollard, 1994).

Recombinant human (rh)LC-1 and its N-terminal peptide derivatives (amino acids 1–188 and 2–26) mimic anti-inflammatory actions of glucocorticoids, and anti-LC-1 antibodies reverse glucocorticoid effects both *in vivo* and *in vitro* (Flower & Rothwell, 1994). There is growing evidence that LC-1 may inhibit inflammation *in vivo* by preventing leukocyte activation and accumulation at the site of injury. Studies in the mouse have shown that intravenous injection of rhLC-1 and its N-terminal derivatives inhibit several neutrophil-dependent models of acute inflammation such as air pouch infiltration, peritonitis, skin oedema and neutropenia (Cirino *et al.*, 1993; Perretti & Flower, 1993; Perretti *et al.*, 1993). More recently, rhLC-1 peptide 2–26 has been shown to mimic the suppressive actions of agents which inhibit leukocyte interaction with the endothelium, including a monoclonal antibody to a subunit of the leukocyte cell surface β_2-integrin adhesion complex (Harris *et al.*, 1995).

The majority of LC-1 identified in peripheral blood leukocytes is present in monocytes and neutrophils (Morand *et al.*, 1995). Maturation of blood leukocytes to tissue macrophages may influence the steroid sensitivity of LC-1 expression, as glucocorticoid-induced increases in LC-1 in alveolar macrophages, although not in blood leukocytes, are accompanied by inhibition of prostaglandin E_2 (PGE_2) release (De Caterina *et al.*, 1993). Other studies have implicated LC-1 in glucocorticoid suppression of PGE_2 release from macrophages (Flower, 1988) and the A549 human adenocarcinoma cell line (Croxtall & Flower, 1992; 1994). Transfection of A549 cells with antisense DNA for an N-terminal portion of LC-1 not only blocks glucocorticoid suppression of PGE_2 release, but also prevents the glucocorticoid-induced cell surface expression of newly synthesized LC-1 (Croxtall & Flower, 1994). Glucocorticoid-induced externalisation of LC-1 has also been reported in a macrophage cell line (Wu *et al.*, 1995), *ex vivo* preparations of rat leukocytes (Browning *et al.*, 1990; Peers *et al.*, 1993) and human monocytes (Goulding *et al.*, 1990a). In some instances, the inhibitory actions of LC-1 occur in parallel with its externalisation, suggesting an extracellular site of action for the protein (Croxtall & Flower, 1994; Wu *et al.*, 1995). Indeed, specific, saturable binding sites for LC-1 have been identified on the surface of monocytes and neutrophils (Goulding *et al.*, 1990b), and surface-bound LC-1 may inhibit IgG interaction with these cells (Goulding & Guyre, 1993).

The ability of glucocorticoids to inhibit release of tumour necrosis factor (TNF)α and PGE_2 from leukocytes in response to various stimuli, including lipopolysaccharide (LPS), is well documented (Beutler *et al.*, 1986; Flower, 1988; Han *et al.*,

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1990). Although release of TNFα from PBMC is important in activation of local inflammatory responses such as neutrophil migration (Baumann & Gauldie, 1994), there is little information on the effects of LC-1 on cytokine release from PBMC. In this study, we examine a role for LC-1 as an inhibitor of LPS- and interleukin (IL)-1β-stimulated secretion of TNFα and PGE_2 from adherent cells isolated from human blood (peripheral blood mononuclear cells, PBMC).

Methods

Preparation and incubation of PBMC

Human blood was collected into 3.2% aqueous trisodium citrate (10 ml per 100 ml blood) under sterile conditions, and diluted two fold with RPMI-1640 medium with 2 mM L-glutamine and gentamycin 10 μg ml^{-1}. Volumes of diluted blood were layered onto Ficoll-Hypaque (<20 pg ml^{-1} endotoxin; Pharmacia, Sweden) and centrifuged at 1200 *g* for 25 min at 22°C. The leukocyte layer was removed, diluted three fold with ice-cold RMPI-1640 medium supplemented with 2% heat inactivated foetal calf serum (FCS) and centrifuged at 1200 *g* for 10 min at 4°C. The cell pellets were resuspended in a smaller volume of ice-cold RMPI-1640 medium (with 2% FCS) and centrifuged as before. This washing step was repeated three times before the cells were resuspended in 10 ml medium (with FCS) and aliquots taken to count cell number in a haemocytometer and to assess cell viability by exclusion of (0.1%) Trypan blue. The suspension was diluted with medium to the required seeding density (1 to 5×10^6 cells ml^{-1}) and plated out into either 96-well cell culture plates (200 μl aliquots) or 24-well cell culture plates (1 ml aliquots). The cells were maintained under a humidified atmosphere of 5% CO_2, 95% air at 37°C for 2 h. The non-adherent cells were washed away by repeated aspiration and replacement with fresh medium, leaving a population of cells which were predominatly (>85%) monocytes. After overnight incubation at 37°C, the adherent cells were washed as before, and medium with drugs or vehicle added. After further incubation, medium was removed from the cells to a 96-well microtitre plate (EIA immunoassay plate; Costar, U.S.A.) either prepared for TNFα assay (see below), or for storage at −20°C until required for PGE_2 assay.

Assay of TNFα

Ninety-six well plates were incubated overnight at 4°C with a 1:5000 dilution of polyclonal sheep anti-human TNFα antibody in coating buffer (0.5 M $NaHCO_3$-Na_2CO_3 buffer, pH 9.6 with 0.02% w/v sodium azide as preservative) or coating buffer alone (to estimate non-specific binding (NSB)). Plates were washed three times with a PBS-Tween-BSA (PBST) buffer (137 mM NaCl, 2 mM KH_2PO_4, 20 mM Na_2HPO_4, 0.05% v/v Tween-20; 0.1% BSA; pH 7.4) and incubated with 1% BSA in coating buffer for 30 min at 22°C. Subsequent incubations were interspersed with extensive washes in PBST; 100 μl or 200 μl (depending on the requirement of sample for PGE_2 assay) of sample or standard (or medium alone, NSB) was added to wells and plates incubated overnight at 4°C. Recombinant human TNFα diluted in medium over the range 0.01 to 5 ng ml^{-1} was assayed in triplicate. A 1:5000 dilution of anti-human TNFα rabbit polyclonal antibody in PBST was added to wells and the plates incubated at 22°C for 2 h, before further incubation with a 1:4000 dilution of goat anti-rabbit IgG horseradish peroxidase conjugate (affinity purified; ICN Biochemicals, U.K.) in PBST (with 1% BSA) for 2 h at 22°C. Substrate (4% urea hydrogen peroxide solution) was added to chromogen (0.1 mg ml^{-1} tetramethylbenzidine in 0.1 M sodium acetate trihydrate; 0.1 M citric acid to pH 6) in the ratio 1:250 and 200 μl added to all wells. Plates were incubated in the dark for 30 min at 22°C and the reaction stopped by addition of 50 μl of 2 M sulphuric acid. Optical density (OD) of chromogen was measured at 450 nm with a SLT plate reader (SLT, Austria) linked to a computer running Soft 2000 Software (Tecan, U.K.). This software was used to construct standard curves and read off sample OD values to calculate TNFα concentration. The limit of assay sensitivity (the mean value for 0 pg ml^{-1} TNFα + 2.5 × standard deviation of mean, $n = 4$ to 6 per assay) varied between 25 and 30 pg ml^{-1} TNFα, with medium from PBMC diluting out in parallel to TNFα standards up to 5000 pg ml^{-1}.

Assay of PGE_2

The concentration of PGE_2 in the incubation medium from human PBMC was measured by a highly specific radioimmunoassay (RIA) which has been previously described (Haworth & Carey, 1986). Briefly, PGE_2 standards were assayed in triplicate and (100 μl) samples in duplicate with a 1:10,000 dilution of anti-PGE_2 antibody. Dextran-coated charcoal was used to separate bound radiolabelled PGE_2 (0.005 μCi per tube [^{3}H]-PGE_2, specific activity 200 Ci $mmol^{-1}$, Amersham, U.K.) from free and, after addition of scintillant (PCS scintillant, Amersham, U.K.) to the supernatant, radioactivity was counted for 4 min using a rack beta counter (LKB, Finland) programmed with an appropriate quench curve. The RIA was routinely sensitive to 5 pg ml^{-1} PGE_2 and linear over 10 to 1000 pg ml^{-1} PGE_2, with medium from PBMC diluting out in parallel to PGE_2 standards over this range.

Immunoblotting for LC-1

After incubation in serum-free medium (RPMI-1640 medium supplemented as described above but without FCS), PBMC were harvested into HEPES-buffered Tyrode solution (composition, mM: NaCl 140, KCl 5, $MgCl_2$ 3, glucose 10, HEPES 5, pH 7.2), washed twice by centrifugation at 5000 *g* for 2 min, solubilized in dissociation buffer (70 mM SDS; 20% glycerol; 1.5% 2-mercapto-ethanol; 0.01% bromophenol blue; 50 mM Tris/4 M HCl, pH 8.3) and heated to 80°C for 2 min. Any cells were removed from medium taken from PBMC (1.5 ml from 24-well plates) by centrifugation at 5000 *g* for 2 min, the supernatant fractions desalted by dialysis (5 kDa exclusion tubing in 3 volumes of 5 litres distilled water over 6 h at 4°C), concentrated by freeze-drying under vacuum for 20 h and solubilized in dissociation buffer as described above. Samples were loaded on to 12.5% polyacrylamide slab gels and separated by electrophoresis (SDS PAGE). Following separation, proteins were transferred to nitrocellulose membrane using a Fast Blot B34 electroblotter (Biometra, Germany), washed in Tris-buffer saline (TBS:20 mM Tris/HCl pH 7.5; 0.5 M NaCl), incubated for 1 h at 22°C in TBS with 3% gelatin and then incubated overnight at 22°C with a 1:200 dilution of anti-LC-1 polyclonal antibody in TBS with 1% gelatin. After washing in TBS, blots were incubated with a 1:2500 dilution of goat anti-rabbit IgG horseradish peroxidase conjugate (BioRad, U.S.A.) in TBS (with 1% gelatin) for 2 h at 22°C and immunoreactive protein was visualized either by incubation in substrate solution (60 mg BioRad HRP colour reagent in 20 ml ice-cold methanol added to 60 μl 30% hydrogen peroxide solution in 100 ml 20 mM TBS) or by enhanced chemiluminescence (ECL; Amersham). Immunoblots were scanned with a laser densitometer (Desage, Germany) and immunoreactive band intensity measured either by reflectance (blots) or absorbance (ECL films) at 580 nm.

Materials

Unless stated otherwise, cell culture media and apparatus were obtained from Gibco Laboratories, Paisley, U.K., and other reagents from the Sigma Chemical Co., Poole, U.K. Antibodies recombinant human TNFα (British Biotechnology, Cambrige, U.K.) were raised in sheep and rabbits, antibodies to PGE_2 in rabbits (Haworth & Carey, 1986), and were used as whole plasma fractions of blood from immunized animals

collected into 3.2% aqueous trisodium citrate (10 ml per 100 ml blood). A fragment of recombinant human lipocortin-1 consisting of amino acids 1–188 (LC-1 fragment) was synthesized as described by Carey *et al.* (1990), and comprised 0.42 mg ml^{-1} protein (>99% purity; <12.5 endotoxin units ml^{-1} in the *Limulus* lysate assay, TechGen, U.K.) in 25 mM Tris, pH 8. LC-1 fragment was conjugated to bovine thyroglobulin using N-(dimethylaminopropyl)-*N'*-ethylcarbodiimide, dialysed, concentrated by freeze-drying, reconstituted in adjuvant and injected (intramuscularly) into the hind limb of a New Zealand white rabbit in a manner similar to that described by Forder & Carey (1983). Anti-LC-1 antibodies (a whole blood plasma fraction from an immunized rabbit) were diluted in sterile phosphate-buffered saline (PBS, pH 7.4) before use. For immunoblotting, rhLC-1, recombinant human annexin-2, and purified human annexin-3 were generously donated by Dr J. Browning (Biogen, Boston, U.S.A.). Recombinant human annexin-5 and a recombinant human annexin mixture (LC-1, annexins 2, 4, 6 and p11) were purchased from Zymed Laboratories (San Francisco, U.S.A.).

Recombinant human (rh)IL-1β (3×10^7 iu mg^{-1}; <0.3 endotoxin units mg^{-1}) donated by Dr A. Shaw (Glaxo, Switzerland), and a single batch of bacterial endotoxin from *Escherichia coli* L3012 (phenol extract), referred to here as lipopolysaccharide (LPS), were diluted in sterile RPMI-1640 medium (<0.5 endotoxin units ml^{-1}) with 2% heat inactivated FCS. Dexamethasone 21-phosphate (disodium salt) was diluted in sterile PBS to the required dexamethasone equivalent concentration.

Statistical analysis

Assay data are expressed as mean ± the standard error of the mean (s.e.mean), with the number of observations in each group indicated in parentheses i.e. ($n=$). Comparison data from two treatment groups was made by Student's two-tailed *t* test. When comparison of data from more than two treatment groups was required, one or two factor analysis of variance (ANOVA) was used as appropriate. Scheffe's multiple comparison test was used *post hoc* to one-way ANOVA. A two-tailed probability of less than 5% (i.e. $P<0.05$) was taken as statistically significant. If the concentration of TNFα in medium from control incubates was below the limit of assay sensitivity, this treatment group was excluded from the statistical analysis.

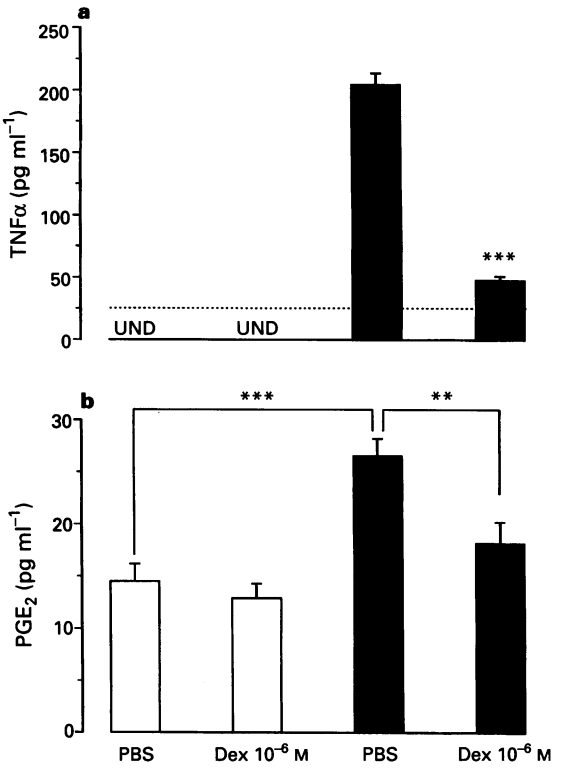

Figure 1 Dexamethasone suppression of LPS-stimulated release of TNFα and PGE_2 from PBMC: PBMC (10^6) were pre-incubated with 10^6 M dexamethasone (Dex) or vehicle (PBS) for 15 min, before addition of 10 μg ml^{-1} LPS (solid column) or medium (open column) and a further 4 h incubation. Medium from cells was assayed for (a) TNFα and (b) PGE_2 content as described in methods. Values shown are means ± s.e.mean ($n=8$). In (a), Dex inhibited LPS-stimulated release of TNFα (***$P<0.001$, Student's unpaired *t* test), assay sensitivity (dotted line) was 25 pg ml^{-1} (UND denotes TNFα concentrations were below assay detection limit). In (b), LPS stimulation of PGE_2 release (***$P<0.001$, two-way ANOVA) was inhibited by Dex (**$P<0.01$, two-way ANOVA).

Results

LPS (10 μg ml^{-1}) stimulation of TNFα release from PBMC was inhibited by 77% ($P<0.001$) when cells were pre-incubated for 15 min, and then co-incubated for 4 h with 10^{-6} M dexamethasone (Dex) (Figure 1a). TNFα release from PBMC incubated for 4 h in the absence of LPS was below the limit of assay sensitivity, 25 pg ml^{-1} (Figure 1a). LPS caused a two fold increase ($P<0.001$) in PGE_2 release from the same cells, which Dex inhibited by 30% ($P<0.01$) (Figure 1b). After 4 h incubation with 10^{-9} M to 10^{-7} M LC-1 fragment or vehicle (Tris) alone, TNFα concentrations in medium from PBMC were below assay sensitivity, 25 pg ml^{-1} (Figure 2a) and PGE_2 concentrations were not significantly altered ($P>0.05$) (Figure 2b). LPS-stimulated TNFα release from PBMC was not significantly altered ($P>0.05$) by the presence of 10^{-9} M to 10^{-7} M LC-1 fragment (Figure 2a), nor did these concentrations of LC-1 fragment inhibit LPS-stimulated ($P<0.001$) release of PGE_2 from the same cells (Figure 2b). In an additional experiment (data not shown), 10^{-9} M to 10^{-7} M LC-1 fragment failed to inhibit the release of TNFα and PGE_2 from PBMC stimulated with 10 ng ml^{-1} LPS.

The cross-reactivity of a 1:200 dilution of anti-LC-1 polyclonal antibody with rhLC-1, human annexins 2 to 6 and the annexin-2 subunit p11 was assessed by immunoblotting (Figure 3). Anti-LC-1 antibody did not detect 50 ng of purified annexin-3 (lane A), recombinant human annexin-2 (lane C) or recombinant human annexin-5 (lane F), but did detect 50 ng of rhLC-1 and LC-1 fragment (lanes D and E). When a mixture of rhLC-1, human annexins 2, 4, 6 and the annexin-2 subunit p11 was immunoblotted, a single immunoreactive species was detected (lane B) with a molecular mass equivalent to that of rhLC-1 (lane D).

After 4 h incubation with Dex or vehicle (PBS), PBMC cellular protein (Figure 4a) and extracellular medium (Figure 4b) were immunoblotted with a 1:200 dilution of anti-LC-1 fragment antibody. Immunoblotting of PBMC cellular protein revealed, in addition to immunoreactive (ir)LC-1 (the upper band in Figure 4a), an immunoreactive species with a molecular mass approximately 7 kDa less than that of irLC-1 (the lower band in Figure 4a). Incubation of PBMC with Dex did not enhance the band intensity for irLC-1 when compared to PBS, although the band intensity for the lower mass immunoreactive protein was increased three fold by the presence of Dex (Figure 4a). Immunoblotting of serum-free medium taken off the same PBMC revealed the presence of irLC-1 only in medium from cells incubated with Dex (Figure 4b). In addition, medium from Dex-treated PBMC contained the lower molecular mass immunoreactive species described above, and an immunoreactive species with a molecular mass approximately 6 kDa greater than that of irLC-1 (Figure 4b). Similar results were obtained when PBMC and extracellular medium were immunoblotted for LC-1 content after 18 h incubation with or without Dex (data not shown).

LPS-stimulated TNFα release ($P<0.001$) from PBMC was suppressed by 73% ($P<0.001$) by Dex, although inclusion of a

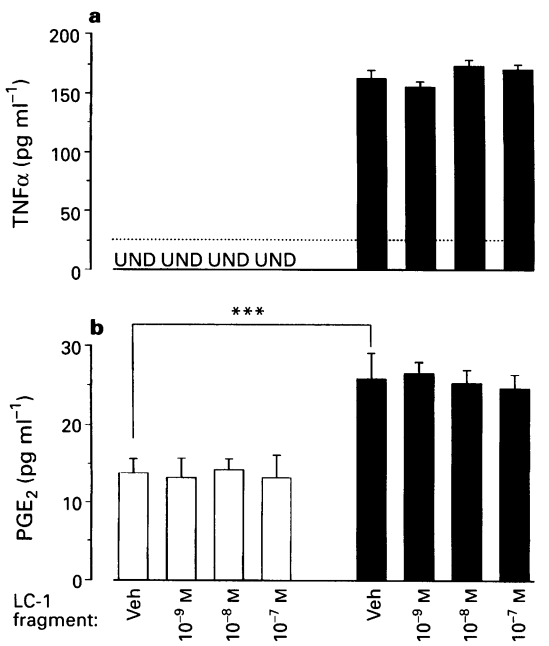

Figure 2 LC-1 fragment does not inhibit LPS-stimulated release of TNFα and PGE_2 from PBMC: PBMC (10^6) were pre-incubated with 10^{-9} M to 10^{-7} LC-1 fragment or vehicle (Tris) for 15 min, before addition of 10 μg ml^{-1} LPS (solid column) or medium (open column) and a further 4 h incubation. Medium from cells was assayed for (a) TNFα and (b) PGE_2 content as described previously. Values shown are means ± s.e.mean ($n=8$). In (a), LC-1 fragment failed to inhibit LPS-stimulated TNFα release ($P>0.05$, one-way ANOVA), assay sensitivity (dotted line) was 25 pg ml^{-1} (UND denotes TNFα concentrations were below assay detection limit). In (b), LPS stimulation of PGE_2 release (***$P<0.001$, one-way ANOVA) was not significantly altered by LC-1 fragment ($P>0.05$, one-way ANOVA).

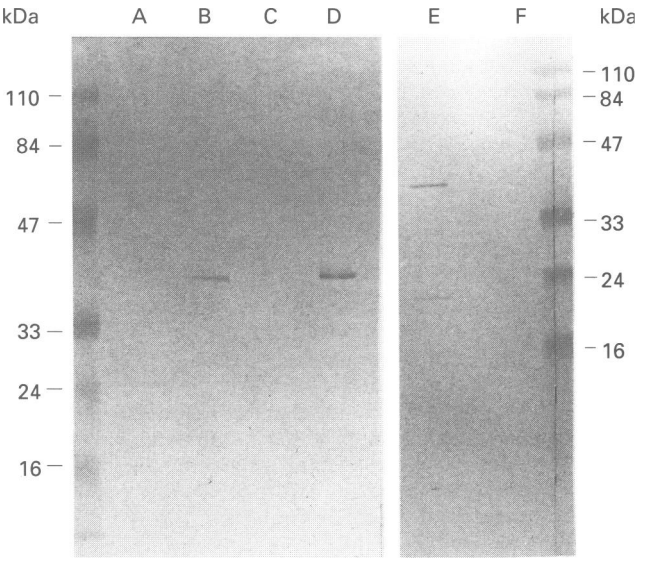

Figure 3 Cross-reactivity of anti-LC-1 fragment polyclonal antibody with rhLC-1 and human annexins 2 to 6: rhLC-1 and recombinant human or purified human annexins 2 to 6 were immunoblotted with 1:200 dilution of anti-LC-1 fragment polyclonal antibody as described in methods. Lanes were (A) 50 ng purified annexin-3; (B) 25 ng each of rhLC-1, recombinant annexins 2, 4, 6 and the annexin-2 subunit p11; (C) 50 ng annexin-2; and (D) 50 ng rhLC-1. On a separate immunoblot lanes were (E) 50 ng each of rhLC-1 and LC-1 fragment; and (F) 50 ng recombinant annexin-5. Molecular weight marker proteins are as indicated. For each lane, the results shown are representative of at least two immunoblotting experiments using anti-LC-1 fragment antibody.

1 : 10,000 (final) dilution of anti-LC-1 fragment antibody in the medium partially reversed ($P<0.001$) this inhibition to 26% (Figure 5a). When a 1 : 10,000 dilution of control antibody was included in the medium, Dex suppression (64%) of LPS-stimulated TNFα release was not significantly altered ($P>0.05$) (Figure 5a). LPS-stimulated release of PGE_2 from the same cells ($P<0.001$) was inhibited by 54% ($P<0.001$) by Dex, and this suppression was reduced ($P<0.001$) to 8% when anti-LC-1 fragment antibody was present in the medium (Figure 5b). Co-incubation with an equivalent dilution of control antibody did not significantly alter ($P>0.05$) Dex suppression (50%) of LPS-stimulated PGE_2 release from cells.

The effect of LC-1 fragment on rhIL-1β-stimulated release of TNFα and PGE_2 from PBMC was investigated. After 18 h, mean TNFα concentration in medium from vehicle-treated PBMC (41 pg ml^{-1}) was reduced to below the limit of assay sensitivity (30 pg ml^{-1}) in the presence of 10^{-9} M or 10^{-7} M LC-1 fragment alone (Figure 6a). A separate study (data not shown) demonstrated that neither 10^{-7} M LC-1 fragment nor its vehicle (Tris), altered the ability of the TNFα assay to detect human TNFα over the standard curve range of 10 to 5000 pg ml^{-1}. After 18 h, 5×10^{-9} M and 5×10^{-8} M rhIL-β enhanced TNFα secretion from PBMC approximately two fold ($P<0.01$ and $P<0.001$ respectively) (Figure 6a). There was no stimulation of TNFα release from PBMC using the above concentrations of rhIL-1β after 4 h, and the apparent rhIL-1β-stimulation of TNFα secretion was not stastically significant at 6 or 12 h ($P>0.05$, data not shown). Co-incubation of rhIL-1β-stimulated PBMC with 10^{-9} M and 10^{-7} M LC-1 fragment rendered TNFα undetectable (Figure 6a). PGE_2 release from unstimulated PBMC (after 18 h) was considerably higher than that seen after 4 h (Figure 6b compared with Figures 1b, 2b and 5b), and was not significantly altered ($P>0.05$) by 10^{-9} M or 10^{-7} M LC-1 fragment. The two fold increase ($P<0.05$) in release of PGE_2 from PBMC stimulated with 5×10^{-8} M rhIL-1β was reversed ($P<0.05$) by 10^{-7} M LC-1 fragment (Figure 6b).

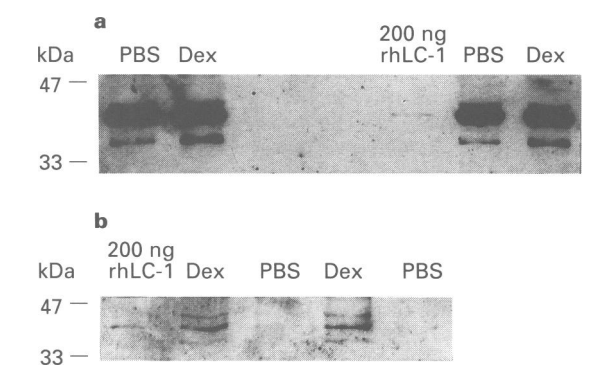

Figure 4 Dexamethasone induces externalization of LC-1 immunoreactivity from PBMC: (a) 10^6 PBMC were incubated in serum-free medium with 10^{-6} M dexamethasone (Dex) or vehicle (PBS) for 4 h. Cells were harvested, pooled (2×10^6), solubilized, cellular proteins (50 μg per track) separated by SDS PAGE and immunoblotted with 1:200 dilution of anti-LC-1 fragment polyclonal antibody, with 20 ng rhLC-1 included on the blot as a control. Duplicates for each treatment and molecular weight markers are as indicated. (b) Medium from the same PBMC was pooled (2×1.5 ml), desalted, concentrated by freeze-drying, extracellular protein solubilized in (200 μl) dissociation buffer and duplicate (100 μl) samples separated by SDS PAGE and immunoblotted as described above. The immunoblots shown in (a) and (b) are both representative of two experiments.

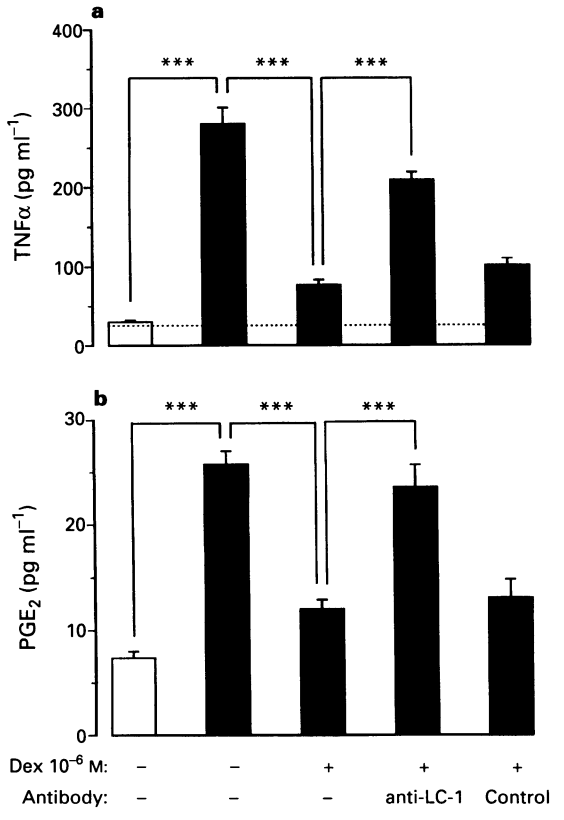

Figure 5 Anti-LC-1 fragment antibody reverses dexamethasone suppression of LPS-stimulated release of TNFα and PGE_2 from PBMC: PBMC (10^6) were pre-incubated with 10^{-6} M dexamethasone (Dex) or vehicle (PBS) and a 1:10,000 (final) dilution of anti-LC-1 fragment or control antibody for 15 min, before addition of 10 μg ml^{-1} LPS (solid column) or medium (open column) and a further 4 h incubation. Medium from cells was assayed for (a) TNFα and (b) PGE_2 content as described previously. Values shown are means ± s.e.mean ($n=6$). Dex suppression (***$P<0.001$, one-way ANOVA) of both LPS-stimulated (a) TNFα release (***$P<0.001$, one-way ANOVA) and (b) PGE_2 release (***$P<0.001$, one-way ANOVA) was partially reversed by anti-LC-1 fragment antibody (***$P<0.001$, one-way ANOVA). Assay sensitivity (dotted line) for TNFα was 25 pg ml^{-1}.

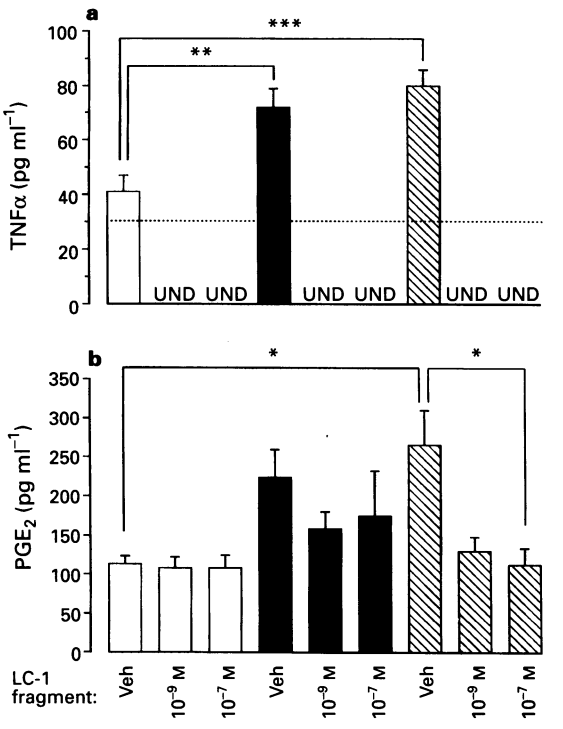

Figure 6 LC-1 fragment suppression of IL-1β-stimulated release of TNFα and PGE_2 from PBMC: PBMC (2×10^5) were pre-incubated with 10^{-9} M or 10^{-7} M LC-1 fragment or vehicle (Tris) for 15 min, before addition of 5×10^{-8} M (hatched column) or 5×10^{-9} M IL-1β (solid column), or medium (open column), and a futher 18 h incubation. Medium from cells was assayed for (a) TNFα and (b) PGE_2 content as described previously. Values shown are means ± s.e.mean ($n=8$). In (a), IL-1β stimulation of TNFα release (**$P<0.01$, ***$P<0.001$, one-way ANOVA) was abolished by LC-1 fragment, assay sensitivity (dotted line) was 30 pg ml^{-1} (UND denotes TNFα concentrations were below assay detection limit). In (b), 5×10^{-8} M IL-1β stimulation of PGE_2 release (*$P<0.05$, one-way ANOVA) was reversed by 10^{-7} M LC-1 fragment (*$P<0.05$, one-way ANOVA).

Discussion

The acute release of pro-inflammatory mediators such as eicosanoids and cytokines from activated tissue macrophages is of primary importance for development of local inflammatory responses. Once released, cytokines such as TNFα act on stromal cells at the reactive site to elicit the release of a secondary wave of cytokines (Baumann & Gauldie, 1994). Several studies have shown the LC-1 and its N-terminal derived peptides can mimic the potent anti-inflammatory actions of glucocorticoids (Flower & Rothwell, 1994), but few have examined the effect of LC-1 on cytokine release. The present study investigated the ability of LC-1 to suppress the release of TNFα and PGE_2 from PBMC stimulated with LPS or rhIL-1β.

Concentrations of TNFα secreted by 'unstimulated' PBMC (i.e. in the absence of LPS or rhIL-1β) were either below or near the limit of assay sensitivity. The very low concentrations of TNFα released from PBMC in the absence of a drug stimulus has been noted previously (e.g. Schindler *et al.*, 1990). Haskill *et al.* (1988) have demonstrated that isolation and adherence of PBMC could induce TNFα gene transcription but that actual secretion of TNFα requires a secondary stimulus such as LPS.

Dex inhibition of LPS-stimulated PGE_2 release from PBMC was significant, but less pronounced than inhibition of TNFα release (Figure 1b). Glucocorticoid suppression of TNFα production from LPS-stimulated PBMC involves inhibition of both TNFα gene transcription and, via a glucocorticoid-inducible protein, mRNA translation (Beutler *et al.*, 1986; Han *et al.*, 1990). Glucocorticoid suppression of PGE_2 production involves inhibition of phospholipase activity (Flower, 1988) and suppression of cyclo-oxygenase-2 (Cox-2) expression at the level of translation (Bailey, 1991) and possibly transcription (Newman *et al.*, 1994).

After incubation with Dex for 4 h (or 18 h, data not shown), LC-1 expression in PBMC was not markedly altered, although that of a lower mass protein was enhanced approximately three fold (Figure 4a). This was accompanied by the Dex-induced appearance of LC-1 (and lower and higher mass species with LC-1-like immunoreactivity) in the extracellular medium (Figure 4b). The high levels of irLC-1 present in PBMC incubated with PBS suggests that LC-1 expression may have already been maximal such that exposure to Dex elicited no clear increase in LC-1 cell content (Figure 4a). Failure of glucocorticoid to induce LC-1 expression in both peripheral blood leukocytes (Morand *et al.*, 1995) and activated monocytes (Browning *et al.*, 1990) has been noted previously. In addition, isolation and adherence of PBMC can induce expression of a range of cellular proteins associated with the acute immune response (Haskill *et al.*, 1988) and Peers *et al.* (1993) have demonstrated a non-glucocorticoid mechanism for increased expression of LC-1 in activated leukocytes from adrenalectomized rats.

Extracellular LC-1 can appear within hours of exposure to glucocorticoid *in vivo* (Peers *et al.*, 1993) and *in vitro* (Croxtall & Flower, 1992; 1994; Peers *et al.*, 1993; Taylor *et al.*, 1993; Wu *et al.*, 1995), as observed here (Figure 4b). There is evidence for translational control of extracellular LC-1 expression by glucocorticoid (Taylor *et al.*, 1993), although Croxtall & Flower (1994) have shown glucocorticoid up-regulation of A549 cell surface LC-1 involves *de novo* synthesis of the protein. Several studies have shown that inhibitory effects of glucocorticoid on cells and tissue *in vitro* are accompanied by the externalization of endogenous LC-1 (Croxtall & Flower, 1992; Taylor *et al.*, 1993; Wu *et al.*, 1995). Exogenous LC-1 (and its N-terminal peptide derivatives) can mimic glucocorticoid suppression, and anti-LC-1 antibodies inhibit glucocorticoid effects in these studies. Thus a pericellular 'pool' of endogenous LC-1 may mediate glucocorticoid actions (Croxtall & Flower, 1992). In the present study, anti-LC-1 fragment antibody (which neutralises glucocorticoid actions both *in vivo* and *in vitro*, Flower & Rothwell, 1994) reversed Dex suppression of LPS-stimulated release of TNFα and PGE_2 from PBMC (Figure 5a, b). Thus endogenous LC-1 may mediate, at least in part, Dex suppression of TNFα and PGE_2 release from PBMC, and this inhibition may be associated with externalization of LC-1 (Figure 4b). Although the mechanism for this inhibition is not yet known, others have demonstrated surface binding sites for LC-1 on mouse and human monocytes (Goulding *et al.*, 1990b; Perretti *et al.*, 1993) which may be important for the immunosuppressive activity of the protein (Goulding & Guyre, 1993; Perretti *et al.*, 1993).

When human rhLC-1 and annexins 2 to 6 were immunoblotted with an anti-LC-1 fragment polyclonal antibody, only rhLC-1 was detected (Figure 3). However, immunoblotting PBMC cellular protein or extracellular medium revealed in addition to LC-1, an immunoreactive species which was 7 kDa lighter (Figure 4a, b). Immunoblotting experiments by others have identified lower molecular mass species with LC-1 like immunoreactivity in the subcellular fractions from leukocytes (Smith *et al.*, 1990; Peers *et al.*, 1993). Findings of previous studies suggest that these LC-1-like proteins are the products of proteolytic degradation of full sequence LC-1 (Flower, 1988). Proteolysed irLC-1 has been detected in macrophage cell lysates despite the presence of protease inhibitors (Peers *et al.*, 1993), and *in vivo*, proteolysis may represent a permissive mechanism for the inhibition of the anti-inflammatory effects of LC-1 (Smith *et al.*, 1990). As to the identity of the higher mass immunoreactive species present in extracellular medium (with an estimated molecular mass 6 kDa greater than irLC-1) (Figure 4b), other tissue/cell immunoblotting studies in the past have detected similar mass species with LC-1-like immunoreactivity (Pepinsky *et al.*, 1989; Ando *et al.*, 1991), including a study in which macrophage-like (U937 cell line) cell lysate was blotted with anti-LC-1 fragment antibody (Murphy *et al.*, 1992). LC-1 is known to cross-link with itself to form a variety of higher molecular mass species, and it has been suggested that the 40–45 kDa species detected in these published studies may result from cross-linking of native LC-1 with another unidentified protein or a fragment derived from *in situ* proteolysis of native LC-1 (Pepinsky *et al.*, 1989; Ando *et al.*, 1991; Murphy *et al.*, 1992). In Figure 4b, the differences in the estimated mass of upper and lower species in comparison to irLC-1 suggest at least part of the peptide fragment that was cleaved off to produce the lower mass species, may have associated with the native protein to produce the higher mass species.

LC-1 has been implicated as a mediator of glucocorticoid inhibition of PLA_2 activity (Flower, 1988), although the specificity of LC-1 inhibition of phospholipase activity, in particular PLA_2 inhibition, is controversial (Raynal & Pollard, 1994). Parente *et al.* (1984) demonstrated that partially-purified lipocortins (39 to 44 kDa) inhibited PGE_2 secretion from leukocytes stimulated with heat-killed *Bordetella pertusis*. More recently, Bailey (1991) has suggested that LC-1 mediates the translational component of glucocorticoid suppression of Cox-2 expression, although this does not seem to be the case in A549 cells (Newman *et al.*, 1994) or a macrophage cell line (Wu *et al.*, 1995). In addition, Morand *et al.* (1993) reported that LC-1 did not inhibit LPS-stimulated IL-1β release from human monocytes.

In the present study, we failed to demonstrate LC-1 fragment inhibition of (10 $\mu g\ ml^{-1}$) LPS-stimulated TNFα or PGE_2 release from PBMC (Figures 2a, b). The discrepancy between the findings of the present study and those of Parente *et al.* (1984) may be attributed to differences in cell preparation, culture conditions, use of LC-1 fragment instead of purified LC-1 or the nature of the stimulus. However, as (10^{-7} M to 10^{-9} M) LC-1 fragment also failed to inhibit stimulation of TNFα and PGE_2 release from PBMC when LPS was used at a thousand fold less concentration (10 $ng\ ml^{-1}$, data not shown), it seems unlikely that lack of inhibition was due to a dose-related effect of LPS. Although LC-1 fragment is known to mimic many of the actions of rhLC-1 (Flower & Rothwell, 1994), both agents exhibit different activity profiles in several *in vivo* and *in vitro* models of inflammation (Perretti *et al.*, 1993; Flower & Rothwell, 1994; Perretti, 1994). In experiments involving cytokine-stimulated leukocyte activation and accumulation, LC-1 fragment has been shown to be considerably less potent than rhLC-1 (Peretti, 1994) and in one study in particular, LC-1 fragment, unlike rhLC-1, exhibited only partial inhibition of IL-1β-induced neutrophil accumulation (Perretti *et al.*, 1993). Thus, we cannot exclude the possibility that rhLC-1 may have demonstrated at least some inhibitory effect on LPS stimulation of TNFα and PGE_2 release, although limited availability of rhLC-1 precluded its use in these types of experiments.

Without knowledge of the mechanism by which, as implied by the antibody reversal data (Figures 5a, b), endogenous LC-1 inhibits LPS-stimulated TNFα and PGE_2 release, it is difficult to say precisely why LC-1 fragment was without effect in this regard, although others have suggested LC-1 fragments may exhibit inconsistent activity because of N-terminal heterogeneity (Perretti *et al.*, 1993). Another possible explanation is that the mechanism by which LC-1 fragment inhibits IL-1β-stimulated TNFα and PGE_2 release (Figures 6a, b) is different from that by which endogenous LC-1 inhibits LPS stimulation. Perretti (1994) has suggested a model in which, LC-1 binds to the surface of human leukocytes via a C-terminal region (amino acids 246 to 254) in order to exert its anti-inflammatory actions. This region is absent from LC-1 fragment and implies that the LC-1 fragment inhibition of IL-1β stimulation observed here (Figures 6a, b), may involve a different mechanism.

The magnitude of rhIL-1β-stimulated TNFα release from PBMC, although statistically significant (Figure 6a), was considerably less than that observed with LPS (see above). rhIL-1β has been reported previously to stimulate secretion of TNFα from human PBMC at the same concentrations used in experiments described here (Ikejima *et al.*, 1990), but the concurrent release of IL-6 may feedback to inhibit TNFα secretion (Schindler *et al.*, 1990). IL-1β up-regulates PLA_2 activity in various cell types (Dower *et al.*, 1992), and may also stimulate PGE_2 release via induction of Cox-2 expression (Newman *et al.*, 1994). In A549 cells, Newman *et al.* (1994) demonstrated the involvement of LC-1 in glucocorticoid suppression of the stimulatory actions of IL-1β on PGE_2 release, but not Cox-2 expression. Thus, the mechanism for LC-1 fragment inhibition of rhIL-1β-stimulated TNFα and PGE_2 secretion (Figures 6a, b) is, as yet, unclear.

rhLC-1 and its N-terminal derivatives inhibit several neutrophil-dependent *in vivo* models of acute inflammation, although this inhibition is not stimulus-specific and occurs in response to various inflammatory stimuli including IL-1, IL-6 and IL-8 (Perretti & Flower, 1993; 1994). Injection of LC-1 directly into the site of inflammation is not as effective, but passive immunisation of mice with anti-LC-1 antiserum abolishes glucocorticoid inhibition of cytokine-stimulated neutrophil migration, suggesting that LC-1 inhibits leukocyte activation prior to tissue infiltration (Perretti & Flower, 1993;

1994). rhLC-1 peptide 2–26 mimics the inhibitory effects *in vivo* of a monoclonal antibody to the CD11b β_2-integrin adhesion complex subunit and other agents that prevent leukocyte margination and adhesion, suggesting that LC-1 suppresses neutrophil migration by interfering with the adhesion cascade during leukocyte activation (Harris *et al.*, 1995). This inhibition of leukocyte activation by extracellular LC-1 may explain the suppressive actions of the protein in other models of acute inflammation (e.g. Cirino *et al.*, 1989).

In the current study, we have shown that glucocorticoid induces the externalization of LC-1 from PBMC, and propose that this endogenous extracellular LC-1 mediates glucocorticoid inhibition of LPS-stimulated PGE_2 and TNFα release from the cells. Whether this represents another facet of LC-1 inhibition of leukocyte activation is not yet clear, although preliminary evidence suggests that LC-1 may also suppress release of these pro-inflammatory mediators in response to other stimuli such as IL-1β.

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